

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
28 August 2003 (28.08.2003)

PCT

(10) International Publication Number  
**WO 03/070026 A1**

(51) International Patent Classification<sup>7</sup>: **A23L 3/32**,  
3/3463, 3/3508

(21) International Application Number: PCT/EP03/01922

(22) International Filing Date: 25 February 2003 (25.02.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
02075929.6 25 February 2002 (25.02.2002) EP

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: PRESERVATION OF LIQUIDS

(57) Abstract: The present invention relates to a liquid which have been treated with an electroporation method and which comprises an effective amount of our antifungal compound.



**WO 03/070026 A1**

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## PRESERVATION OF LIQUIDS

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### Field of the invention

The present invention relates to a process for preserving liquids, e.g. beverages, against spoilage by fungi.

### Background of the invention

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The prevention of fungus growth is an important topic to the food industry. Fungal spoilage can lead to serious economic losses. Some food products are very susceptible to fungal growth. Beverages such as fruit juices, lemonades, wine, ice tea and beer are examples of such food products. Spoilage by fungi does not only affect the quality of the product, but also represents a health risk. It is well known that some fungus species, which grow in beverages, can produce mycotoxins. Some mycotoxins are extremely dangerous because they can induce cancer. Therefore the presence of unwanted fungus species in food products should always be prevented.

15

Up to now microorganisms present in food products, such as beverages, are mostly eliminated by a heat treatment, e.g. pasteurization or sterilization. However such treatment affects the quality of the product. Chemical and physical properties of the product will change in a negative way causing loss of nutritional value, organoleptic properties and colour amongst others. No methods of preventing spoilage of food products, especially by fungus species, without affecting the quality of the food product in a negative way are presently known.

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### Description of the invention

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The object of this invention is to provide microbially safe liquid food products without alteration of its original characteristics, such as colour, texture, flavour, odour and nutritional value. According to the invention the liquid foodstuff is treated with an electroporation method, such as Pulsed Electric Field, and an antifungal agent which prevents spoilage of said products by fungi, is added to the liquid foodstuff. Surprisingly we found that the pulsed electric field process is effective against vegetative cells and the antifungal composition prevents spoilage caused by germination and outgrowth of fungal spores. Preservatives, which prevent the outgrowth of bacterial spores, may also be

added. The process of the invention is conducted at minimal elevated temperatures and has an antimicrobial effect against spoilage organisms, including heat-resistant fungi, without affecting the sensory and nutritional characteristics of the liquid.

The present invention provides a liquid which has been treated with an electroporation method and which comprises an effective amount of an antifungal compound, an electroporated liquid comprising an effective amount of an antifungal compound, and/or a liquid which is microbially stable for at least 30 days and is substantially free of sensory effect induced by a heat treatment or has a minimal sensory effect compared with the effect induced by heat treatment, and containing an amount of antifungal agent.

The quality of PEF-treated product depends on the inactivation level of the target micro-organism(s) and on the sensorial and nutritional properties which mainly depend on the temperature reached during the treatment.

A PEF-treatment is called a mild-treatment when the temperature reached during the PEF-treatment stays below the temperature that induces changes of the product properties, or below the temperature usually attained by conventional heat treatment (pasteurisation). A PEF-treatment or an antifungal compound can induce total inactivation of the target microorganism(s) or sublethal damage (partial inactivation, destabilisation of the microbial structure). However a PEF-treatment will in general not inactivate spores of microorganisms. A sub optimal PEF-treatment or a sub optimal natamycin concentration induce sublethal damage. For example a sub optimal natamycin concentration is a natamycin concentration lower than the MIC value (Minimal Inhibition Concentration).

Suboptimal treatments have sensorial (minimal PEF-temperature) and economic (lower PEF-energy and reduced natamycin concentration) benefits.

For example we have found that orange juice treated with the process of the invention during storage at 4°C retains more vitamin C than heat-treated juice. Moreover, the juice treated according to the invention has a lower browning index than heat-treated juice. PEF-treated juice showed a brighter color than heat-treated juice. And finally the particles of juice treated according to the invention have smaller particles than heat-treated orange juice. In general the process of the invention shows several advantages compared to heat treatment for example in the better retention of Vitamin C, prevention of unwanted maillard reactions and no noticeable differences between liquids treated with the process of the

present invention and untreated liquids. These differences are much smaller than differences between heat treated (pasteurised) liquids and untreated liquids.

The present invention further provides a method of treating a liquid, which  
5 comprises:

- (a) subjecting the liquid to an electroporation method; and
- (b) adding an effective amount of an antifungal compound to the liquid.

The method described in the present invention can be used to prevent the growth  
10 of microorganisms, especially fungi, in a wide variety of liquid products.

An electroporation method is advantageously used in combination with the addition of an antifungal compound to preserve a liquid.

Examples of an electroporation method, which can be used as non-thermal preservation method for liquid food products, are methods using electricity, such as  
15 methods known under the name Pulsed Electric Field (PEF), High Pulsed Electric Field, Low Pulsed Electric Field, High Voltage Arc Discharge and Streamer Plasma. The present invention may utilise any of these methods, which induce electroporation of the membrane of microorganisms. The preferred electroporation method is PEF.

PEF can be applied to fruit juices, lemonades, wine, beer, liquid egg products and  
20 other types of pumpable products.

PEF processing involves the application of pulses of high or low voltage to products placed between two electrodes. The shortness of the pulses attempts to minimize heating of the product. The pulsed electric field device for the treatment of pumpable food products has at least two electrodes for supplying an electric field to the  
25 liquid product. All electrodes include an electrode flow chamber for accepting the flow of the liquid food product and for making electric contact with the liquid product. The PEF treatment device also includes at least one insulator positioned between two consecutive electrodes in order to electrically insulate these two electrodes from each other. The electrode flow chambers and the insulator flow chamber(s) include an inlet aperture and  
30 an outlet aperture. The PEF apparatus may also include a voltage pulse generator for supplying a pulsed electric field of high or low intensity, a PEF liquid product treatment device for subjecting the liquid product to the pulsed electric field and all required annexes such as a tank for storing the product to be treated, a tank to receive the treated

product, a device for removing oxygen and other gas from the liquid, a pump for providing a continuous flow and at least one heat exchanger for regulating the temperature of the liquid product. The PEF treatment device is in communication with the high voltage pulse generator that applies a high voltage signal of variable voltage, frequency, pulse duration, shape and polarity to the electrodes.

The high voltage electric field causes death of the vegetative cells of microorganisms by electroporation or lysis of the microbial cell membrane. Pulsed electric fields treatments are effective at inactivating vegetative cells. We have found that fungus spores will survive the PEF treatment because of the spore's rigid structure and ability to resist unfavourable environmental conditions. As a result, PEF treatment alone can not be used in practice to prevent spoilage of liquids in which fungi can grow. PEF treatment will not prevent the outgrowth of fungal spores, whose occurrence and growth of fungus spores in beverages represents an important problem in the current food industry. More recently, the occurrence of more heat resistant fungus species has caused problems in the food industry.

Several antifungal agents, which are used to prevent outgrowth of fungi in food products, can be used in the process of the present invention. Examples of fungicides are polyene antimycotics (e.g. natamycin, nystatin, lucensomycin or amphotericin B); organic acids (e.g. benzoic acid, sorbic acid, propionic acid and lactic acid); salts of said organic acids (e.g. benzoate, sorbate, propionate and lactate); imidazoles or their salts (e.g. imazalil); or any antifungal agent known in the art. The antifungal composition can also be a combination of two or more of the above-mentioned compounds. The effective amount of an antifungal together with the electroporation method prevents the growth of micro-organisms, especially fungus, in the electroporated liquid product. An effective amount of an antifungal compound can be a suboptimal concentration, the MIC value, or higher.

A sub optimal concentration is preferred.

The present invention discloses the combination of the use of an antifungal composition and together with methods based on the electroporation of the membrane of microorganisms (such as Pulsed Electric Field) to prevent the spoilage of liquids by fungi.

Unexpectedly, we have found that spores of fungus species, especially heat resistant fungus species, are not affected completely by a PEF treatment. A PEF treatment alone even leads to specific selection of these spoilage and/or pathogenic species in food products.

5 Moreover, we have also found that antifungal agents, especially natamycin and sorbate are not inactivated by the PEF treatment. Therefore said agents can be added to the liquid product, e.g. a fruit juice, before executing the PEF treatment. Alternatively the antifungal agent may also be added to the liquid after the PEF treatment. The PEF treatment will inactivate all vegetative cells present in the product. However fungus  
10 spores will not be inactivated. The antifungal agent will prevent spoilage caused by the germination of fungus spores in the liquid. In addition, an antibacterial agent can be added to the product to inactivate bacterial spores. Preferably nisin or lysozyme are used as antibacterial agent.

A preferred fungicide to prevent spoilage of food products by fungi is natamycin.  
15 Natamycin has been used for more than 30 years to prevent outgrowth of fungi on cheeses and sausages. Natamycin is on the market under the brand name of Delvolid® or Actistab®, a powder composition containing 50% (w/w) of natamycin and 50% (w/w) of lactose or glucose respectively. Natamycin has a MIC (Minimal Inhibition Concentration) of less than 10 ppm for most food born fungi while its solubility in water is  
20 from 30 to 50 ppm. Natamycin can easily be applied to prevent spoilage by fungi in beverages by mixing the powder through the liquid. Under normal hygienic conditions for beverages such as fruit juices, wine, beer, ice tea and lemonades a concentration of 1-50 ppm, preferably 3-10 ppm of natamycin is usually sufficient to prevent fungal growth. The effective amount of antifungal compound means the amount of the antifungal  
25 compound needed to prevent fungus growth. A method for determining the minimal effective amount of the antifungal compound is described in Example 1. We have found that natamycin is especially effective against the growth of heat resistant fungus species.

Another preservative that prevents fungus growth is sorbate / sorbic acid. Usually concentrations of 500 – 2000 ppm of sorbate are sufficient to prevent fungal growth.  
30 However, said concentrations will sometimes be insufficient to prevent spoilage of the product because sorbate resistant fungal species may be present. In such cases, higher concentrations are used.

The concentration of the antifungal agent, e.g. natamycin or sorbate can be reduced considerably by combined treatment with PEF. Although of course the exact concentration of the fungicide is determined by many external factors, such as the hygienic conditions, a reduction of at least 50% of the necessary concentration of the antifungal agent can be achieved.

This invention describes for the first time a method to inactivate all microorganisms, especially fungi, present in a liquid without affecting the organoleptic properties, the colour and the nutritional value of the product in a negative way.

The present method is suitable for the treatment of consumable liquids such as fruit juices, wine, beer, lemonades, ice tea, liquid eggs, milk products, desserts and yoghurts. Therefore the consumable liquids are liquids preferably comprising sugars and/or proteins and/or free amino acids. Moreover the present invention can also be used for the treatment of other pumpable liquids such as processing fluid streams, blood, water, ecosystem waters, pharmaceutical products, cosmetics and process water. The term "pumpable products" means any product, which is capable of being pumped or conveyed through pipes or conduits, including solid items conveyed in a conductive aqueous solution. Examples are products obtained from fruits, vegetables and milk such as marmalades, jams, fruit pulp, vegetable extracts, oil, fluid butter and mayonnaise. Alternatively the fluid may also contain pieces of fruit. A heat treatment, e.g. pasteurisation or sterilization of consumable products containing sugars and proteins and/or free amino acids, will result in a change in a negative way of the physical properties which is prevented when PEF-treatment is used instead of the heat treatment. For example Maillard reactions and Maillard reaction products are prevented.

The invention described herein is related to any method based on the electroporation of membranes of microbial cells (such as PEF) in combination with an antifungal compound or combinations of antifungal compounds. Optionally antibacterial agents known in the art may be added. Preferred antibacterial agents used are nisin and lysozyme.

The process of the invention can be performed with any pulsed electric field apparatus independently of the nature of the individual physical components of the PEF device, such as pipes, wire, switches, power supplies, pulser, sensors and computers. Further the process of the invention can be used in any pulsed electric field preservation process, independently of the intensity of the different parameters, such as the electric

field, the frequency, the pulse length, the number of pulses, the pulse shape and polarity and the total energy density applied.

The process of this invention is specifically suitable for inhibition of growth of heat-resistant fungi without causing detrimental effects to the product. Spoilage of beverages, thermally processed fruits and fruit products by heat-resistant fungi has been recognized (Tournas, V. (1994), Heat-resistant fungi of importance to the food and beverage industry, Critical Review for Microbiology, 20, 243-263 and Beuchat, L.R., Rice, S.L. (1979), *Bysschlamys* spp. and their importance in processed fruits, Advances in Food Research, 25, 237-288). *Byssochlamys fulva*, *Byssochlamys nivea*, *Talaromyces macrosporus* have been most frequently encountered. Heat-resistant fungi are characterized by the production of ascospores or similar structures with heat resistance. This enables them to survive the thermal processes given to some beverages. Production of pectic enzymes by *Byssochlamys* can result in complete breakdown of texture in fruit products and also can result in off-flavor development. Some *Byssochlamys* species produce patulin and byssochlamys acid, which both have toxic effects. Heat-resistant fungi, therefore, constitute a public hazard as well as a spoilage problem.

While ascospores of fungi are not inactivated in general by pulsed electric field, addition of an antifungal agent, such as natamycin, to the product to be processed or after processing prevents growth of fungi including heat-resistant fungi.

#### **Example 1: Natamycin MIC-value of heat resistant spoilage fungus species**

This example demonstrates the antifungal effect of natamycin against several important spoilage fungi responsible for many problems in today's food industry.

The minimal inhibition concentration (MIC) of these fungi or the minimal effective amount of the antifungal compound was determined using the agar diffusion method, which is well known in the art. Fungus spores were grown on agar plates containing different concentrations of natamycin. The concentration of natamycin on which no visible growth could be observed was considered as the minimal inhibition concentration for that particular fungus strain. The results are presented in Table 1. The results clearly demonstrate that natamycin at concentrations < 5 ppm inhibits the outgrowth of fungal spores which survived the PEF treatment.



Table I: sensitivity of spoilage fungi towards natamycin

Fungus Species	MIC (ppm)	Isolated from
<i>Byssoschlamys nivea</i> 424.300	< 2,5	-
<i>Mucor circinelloides</i> 366.70	< 2,5	Strawberries
<i>Aspergillus versicolor</i> 245.62	< 2,5	-
<i>Stemphiliomma valparadisiacum</i> 510.83	< 5	Apple juice
<i>Talaromyces macrosporus</i> 130.89	< 2,5	Pineapple juice
<i>Cladosporium tenuissimum</i> 117.79	< 2,5	Fruit
<i>Zygosporium mycophilum</i> 396.49	< 2,5	Apple pulp
<i>Peacilomyces variotii</i> 10274	< 2,5	-

**Example 2: The antifungal effect of natamycin in apple juice**

5 This example describes the activity of natamycin against two fungal species, which are well known for causing spoilage problems in food industry.

All experiments were done with pure apple juice. The chosen concentrations of natamycin were sub-optimal (= below MIC).

At higher concentrations, natamycin fully inhibits the outgrowth of said strains.

10 All experiments were executed in duplo.

a. The effect of natamycin against the spore forming yeast *Zygosaccharomyces bailii* CBS 1097 was examined. Apple juice was inoculated with  $10^4$  Colony Forming Units (CFU)/ml of apple juice. Natamycin was added at a concentration of 1 ppm.

15

b. The effect of natamycin was tested against *Penicillium italicum* ATCC 36041. A spore suspension was prepared using well-known methods. The freshly prepared spore suspension was added to apple juice to a final concentration of  $10^4$  spores/ml of apple juice. Natamycin was added at a concentration of 5 ppm.

20

The samples were incubated at room temperature until fungal growth was visually observed (turbidity of the juice). The results are presented in table 2.

Table 2: Activity of natamycin under sub-optimal conditions

Microorganism	Natamycin Concentration	Time to spoilage
a. <i>Zygosaccharomyces bailii</i>	1.0 ppm	2 days
b. <i>Penicillium italicum</i>	5.0 ppm	4 days

### Example 3: The antifungal effect of a Pulsed Electric Field (PEF) treatment in apple juice

This example describes the effect of a Pulsed Electric Field treatment against the two fungal strains described in example 2.

The chosen PEF-treatment parameters were sub-optimal. At higher energy levels the outgrowth of said strains is fully inhibited.

The pulsed electric fields treatments were applied according to well-known procedures for PEF-treatments of liquids. The apple juice to be treated was pumped through the PEF-treatment chamber with a flow rate of 60 litres per hour. The treatment temperature also called juice temperature before the PEF-treatment chamber was 17°C. The value of the electric field strength and the pulse length kept constant were 35 kV/cm and 2-µs, respectively. Three samples points were validated by variation of the pulse frequency, from 8.6 Hz (treatment A) to 5.7 Hz (treatment B) and 2.9 Hz (treatment C). With the above-mentioned parameters, the apple juice received 12, 8 or 4 pulses; those correspond to an energy density of 71, 37 and 12.5 J/ml of juice, respectively. In these conditions, the temperature of the apple juice did not exceed 34°C, which implies a mild treatment.

All experiments were executed in duplo.

a. A PEF-treatment was applied on *Zygosaccharomyces bailii* at a concentration of 10<sup>4</sup> CFU/ml of apple juice.

b. A PEF-treatment was applied on *Penicillium italicum* at a concentration of 10<sup>4</sup> spores/ml.

The results are presented in table 3.

Table 3: Activity of PEF under sub-optimal conditions

Microorganism	Time to spoilage Treatment A	Time to spoilage Treatment B & C
a. <i>Z. baillii</i>	4 days	2 days
b. <i>P. italicum</i>	6 days	6 days

**Example 4: The antifungal effect of a combined treatment: PEF with natamycin in apple juice**

This example describes the effect of natamycin combined with a Pulsed Electric Field treatment against the fungal strains described in example 2.

The chosen inoculation levels, natamycin concentrations and PEF treatments were as described in the examples 2 and 3. The natamycin can be added before or after the PEF treatment.

All samples were incubated at room temperature until fungal growth was visually observed (turbidity of the juice). The results are presented in table 4.

Table 4: Activity of the combination of natamycin and PEF

Microorganism	Treatment conditions	Time to spoilage	
		Natamycin added after PEF-treatment	Natamycin added before PEF-treatment
a. <i>Z. baillii</i>	PEF-treatment A	not determined	> 30 days
	PEF-treatment B	> 30 days	not determined
b. <i>P. italicum</i>	PEF-treatment C	> 30 days	> 30 days

The above results clearly demonstrate that a combination of low natamycin concentrations and an extremely mild PEF-treatment prevents the spoilage of apple juice. In particular, the data demonstrate that the combined effect is substantially greater than the effect of natamycin or PEF alone.

This example discloses for the first time the production of microbial stable apple juice with hardly any loss of organoleptic or nutritional properties.

#### **Example 5: Stability of natamycin in apple juice during PEF treatment**

This example illustrates the resistance of natamycin to PEF treatments. Natamycin was added to apple juice at a concentration of 5 ppm and submitted to pulsed electric field treatments as described in example 3. The natamycin content after the PEF-treatments was determined by HPLC. None of the analyzed samples showed a decrease in the natamycin concentration.

#### **Example 6: The effect of sorbate against a sorbate-resistant yeast in apple juice**

This example describes the activity of sorbate against the sorbate-resistant yeast *Zygosaccharomyces bailii* CBS 1097.

*Zygosaccharomyces bailii* was inoculated in apple juice as described in example 2. Sorbate was added at a concentration of 800 ppm. The samples were incubated at room temperature until fungal growth was visually observed (turbidity of the juice), which was the case after 6 days of incubation.

All experiments were executed in duplo.

#### **Example 7: The antifungal effect of a combined treatment: PEF with sorbate in apple juice**

This example describes the effect of sorbate combined with a Pulsed Electric Field treatment against the sorbate-resistant yeast *Zygosaccharomyces bailii* as described in example 6.

The PEF-treatment was used as described in the example 3. The treatment parameters were chosen so that the juice temperature did not exceed 26°C (treatment B).

The samples were incubated at room temperature. Fungal growth was not observed for at least 30 days.

It can be concluded that as for natamycin (example 4) also for sorbate in combination with a PEF treatment microbially stable apple juice without hardly any loss of quality can be prepared.

5     **Example 8: The antifungal effect of natamycin against heat-resistant fungus**  
:     **spores in apple juice**

10     This example describes the activity of natamycin against spores of a heat resistant fungal species, which is well known for causing spoilage problems in food industry. All experiments were executed on *Talaromyces macrosporus* CBS 130.89 spores suspended in pure apple juice. The chosen concentration of natamycin was sub-optimal. At higher concentrations natamycin fully inhibits the outgrowth of the strain. All experiment were executed in duplo.

15     Apple juice was inoculated with  $10^4$  spores/ml of apple juice. Natamycin was added at a concentration of 2.5 ppm. The spores suspended in the apple juice were submitted to a heat treatment at 80°C for 5 minutes in order to stimulate their germination.

20     The samples were incubated at room temperature until fungal growth was visually observed (turbidity of the juice). Growth was observed after 4 days of incubation at 25°C.

   All experiments were executed in duplo.

20     **Example 9: The antifungal effect of a Pulsed Electric Field (PEF) treatment against heat-resistant fungus spores in apple juice**

   This example describes the effect of a Pulsed Electric Field treatment against heat-resistant spores of *Talaromyces macrosporus* CBS 130.89.

25     The pulsed electric fields treatments were applied as describe in the example 3. One sample point was validated for a frequency of 8.6 Hz (treatment A). The temperature of the apple juice did not exceed 34°C, which implies a mild treatment. Even a more severe PEF treatment does not lead to fully inactivation of the spores of *Talaromyces macrosporus*.

30     Apple juice was inoculated with  $10^4$  spores/ml of apple juice. Subsequently to the PEF-treatment, the spores suspended in the apple juice were submitted to a heat treatment at 80°C for 5 minutes in order to stimulate their germination.

All experiments were executed in duplo

The samples were incubated at room temperature until fungal growth was visually observed (turbidity of the juice). Growth was observed after 3 days of incubation at 25°C.

5     **Example 10: The antifungal effect on heat-resistant fungus spores of a combined**  
:     **treatment: PEF with natamycin in apple juice**

This example describes the effect of natamycin combined with a Pulsed Electric Field treatment against the fungal strains described in example 8.

10     The chosen inoculation levels, natamycin concentrations and PEF treatments  
were as described in the examples 8 and 9. The natamycin can be added before or after  
the PEF treatment.

All experiments were executed in duplo.

All samples were incubated at room temperature until fungal growth was visually observed (turbidity of the juice). Growth was observed after 9 days of incubation at 25°C.

15     The above results clearly demonstrate that a combination of low natamycin  
concentration and an extremely mild PEF-treatment prevent the spoilage of apple juice  
by heat resistant fungus.

CLAIMS

1. A liquid which has been treated with an electroporation method and which  
5 comprises an effective amount of an antifungal compound.
2. A liquid according to claim 1, which is a food, feed containing liquid or a  
beverage.
3. A liquid according to claim 1 or 2 whereby the electroporation method is a pulsed  
electric field.
- 10 4. A liquid according to any one of claims 1 to 3 where the antifungal compound is  
natamycin or sorbic acid.
5. A liquid according to any one of claims 1 to 4 further comprising an antibacterial  
agent.
6. A liquid according to claim 6 wherein the antibacterial agent is nisin or lysozyme.
- 15 7. A method of treating a liquid, which comprises:
  - (a) subjecting the liquid to an electroporation method; and
  - (b) adding an effective amount of an antifungal compound to the  
liquid.
8. A method according to claim 7 whereby the electroporation method is a pulsed  
20 electric field method.
9. A method according claim 7 or 8 whereby the antifungal compound is natamycin  
or sorbic acid.
10. A method according to any one of claims 7 to 9 whereby the antifungal compound  
is added to the liquid before the electroporation method.
- 25 11. A method according to any one of claims 7 to 10 further comprising the addition  
of an effective amount of antibacterial agent.
12. A method according to claim 11 wherein the antibacterial agent is nisin or  
lysozyme.
13. A liquid obtainable by a method according to any one of claims 7 to 12.
- 30 14. Use of an electroporation method in combination with the addition of an antifungal  
compound to preserve a liquid.
15. The use according to claim 14 where the liquid is a beverage.

16. A liquid comprising sugars and proteins and/or free amino acids which is microbially stable for at least 30 days, free of sensory effect induced by a heat treatment and comprising an amount of an antifungal agent.
17. A liquid according to claim 15 which is a fruit juice, lemonade, wine, beer, preferably a fruit juice and more preferably apple juice.



# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/01922

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A23L3/32 A23L3/3463 A23L3/3508

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A23L A61L A23B A23C C12H C02F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, FSTA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 838 154 A (DUNN JOSEPH E ET AL) 13 June 1989 (1989-06-13) column 21, line 25 -column 23, line 45 abstract; claims column 15, line 52-60	1-17
X	WO 97 05067 A (MILDE HELMUT I ;PHILP SANBORN F (US)) 13 February 1997 (1997-02-13) page 2, line 21 -page 3, line 24; claims; figure	1-3,5,7, 8,10,11, 13-17 4,6,9,12
Y	US 5 895 681 A (CIRIGLIANO MICHAEL CHARLES ET AL) 20 April 1999 (1999-04-20) abstract; claims; examples	4,9
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☒ Further documents are listed in the continuation of box C.

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23 June 2003

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/01922

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHARMA S.: "Development of a protein fortified fruit beverage and its quality when processed with pulsed electric field treatment" JOURNAL OF FOOD QUALITY, vol. 21, no. 6, 1998, pages 459-473, XP008005564	1-3,5,7, 8,10,11, 13-17
Y	the whole document	4,6,9,12
Y	US 5 773 062 A (CIRIGLIANO MICHAEL CHARLES ET AL) 30 June 1998 (1998-06-30) claims	4,9
Y	WO 00 06692 A (ARTHUR GAEL ;GAO YUN CAI (CA); CHARTER EDWARD (CA); KRENTZ SHERI ( ) 10 February 2000 (2000-02-10) abstract	6,12
Y	US 5 514 391 A (BUSHNELL ANDREW H ET AL) 7 May 1996 (1996-05-07) column 5, line 42-45 column 6, line 11-18; claims	1-17
Y	US 5 895 680 A (BUCHANAN MICHELE ALICE ET AL) 20 April 1999 (1999-04-20) claims	1-17
Y	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANKFURT/MAIN, DE; LIN Q L ET AL: "Application of lysozyme in beverages." Database accession no. 96-1-01-h0131 XP002244897 abstract & FOOD SCIENCE, CHINA 1994 DEP. OF FOOD SCI. & TECH., HUNAN AGRIC. UNIV., CHANGSHA, HUNAN 410128, CHINA,	6,12
Y	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANKFURT/MAIN, DE; "Biopreservation of foods using nisin." Database accession no. 2003-00-t0319 XP002244898 abstract & FLAIR-FLOW REPORTS FFE 387/01/SME3, 1P. 2001 CORRESPONDENCE ADDRESS, E. J. SMID, AGROTECH. RES. INST., WAGENINGEN UNIV. CENT., PO BOX 17, NL-6700 AA, NETHERLANDS. TEL. 00 31 317 475000. FAX 00 31 317 475347. E-MAIL M.H.J.BENNIK(A)ATO.DLO.NL,	6,12

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 03/01922

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	HODGINS A.: "Pasteurization of fresh orange juice using low-energy pulsed electric field." JOURNAL OF FOOD SCIENCE, vol. 67, no. 6, 2002, pages 2294-2298, XP002244896 the whole document ----	1-17
A	US 5 776 529 A (BARBOSA-CANOVAS GUSTAVO V ET AL) 7 July 1998 (1998-07-07) column 1, line 66-68; claims 1,15,16 ----	4,9
A	US 5 354 902 A (MERCIADEZ MEL ET AL) 11 October 1994 (1994-10-11) -----	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 03/01922

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4838154	A	13-06-1989	US 4695472 A 22-09-1987
			US 5235905 A 17-08-1993
			AU 598509 B2 28-06-1990
			DE 3689935 D1 28-07-1994
			DE 3689935 T2 29-09-1994
			EP 0340212 A1 08-11-1989
			FI 892535 A 24-05-1989
			JP 2501348 T 17-05-1990
			JP 2781558 B2 30-07-1998
			NO 883258 A 26-09-1988
			WO 8803763 A1 02-06-1988
			US 5048404 A 17-09-1991
WO 9705067	A	13-02-1997	WO 9705067 A1 13-02-1997
US 5895681	A	20-04-1999	US 5773062 A 30-06-1998
			US 5738888 A 14-04-1998
US 5773062	A	30-06-1998	US 5895681 A 20-04-1999
			US 5738888 A 14-04-1998
WO 0006692	A	10-02-2000	AU 5022399 A 21-02-2000
			WO 0006692 A1 10-02-2000
			US 2002001582 A1 03-01-2002
US 5514391	A	07-05-1996	AT 206879 T 15-11-2001
			AU 5183396 A 30-12-1996
			CA 2223509 A1 19-12-1996
			DE 69616050 D1 22-11-2001
			DE 69616050 T2 29-05-2002
			EP 0831729 A1 01-04-1998
			JP 11506011 T 02-06-1999
			WO 9639876 A1 19-12-1996
US 5895680	A	20-04-1999	NONE
US 5776529	A	07-07-1998	US 5662031 A 02-09-1997
			US 6019031 A 01-02-2000
US 5354902	A	11-10-1994	AU 666046 B2 25-01-1996
			AU 4917993 A 12-05-1994
			BR 9304347 A 05-07-1994
			CA 2109028 A1 27-04-1994
			CA 2109150 A1 27-04-1994
			EP 0595576 A2 04-05-1994
			FI 934711 A 27-04-1994
			GR 93100416 A ,B 30-06-1994
			IL 107370 A 10-06-1997
			JP 6205657 A 26-07-1994
			KR 233923 B1 15-12-1999
			MX 9306656 A1 29-04-1994
			MX 9306672 A1 29-04-1994
			NO 933841 A 27-04-1994
			NZ 250011 A 25-11-1994
			PH 30358 A 02-04-1997
			RU 2126680 C1 27-02-1999
			ZA 9307913 A 25-04-1995

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